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(54) Title: CEPHALOSPORIN ESTERASE GENE FROM *RHODOSPORIDIUM TORULOIDES*

(57) Abstract

Nucleic acids coding for cephalosporin esterase from *Rhodosporidium toruloides*. Also, isolated cephalosporin esterase, expression vectors, host cells and a method for production of cephalosporin esterase.

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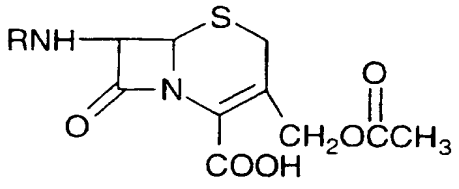
CEPHALOSPORIN ESTERASE GENE FROM *RHODOSPORIDIUM TORULOIDES*

Field of the Invention:

The present invention concerns isolated cephalosporin esterase from *Rhodosporidium toruloides* and nucleic acids encoding said esterase.

Background of the Invention:

Cephalosporin esterase is a general term for an enzyme which is capable of hydrolyzing the 3' acetyl group of cephalosporins of the general structure I to its corresponding desacetyl compound II.



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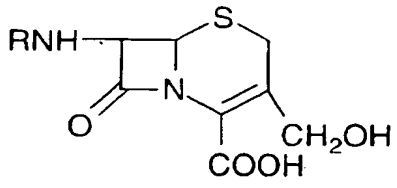

II.

Chemical deacetylation of cephalosporins is performed under extreme pH conditions which generally tend to give side products in addition to the desired desacetyl compound. Enzymatic deacetylation has been described in a number of journal articles and patents. The cephalosporin C esterase activity of the pink yeast *Rhodosporidium toruloides* was first reported by Smith et al. at Glaxo Laboratories U.S. Patent No. 4,533,632 and was used in U.S. Patent No. 5,512,454. However, whole cells or crude extracts were used for the conversion and the enzyme was not purified and characterized.

Heretofore, isolated cephalosporin esterase from *Rhodosporidium toruloides* and nucleic acids encoding the esterase has been unknown.

Summary of the Invention

The present invention is directed to isolated and purified cephalosporin esterase from *Rhodosporidium toruloides* preferably having the sequence of SEQ. I.D. NOS.: 2 or 4. SEQ. ID. NO.: 2 is the amino acid sequence of the entire or intact esterase whereas SEQ. ID. NO.: 4 is the sequence of the mature peptide which is a 551 mamino acid fragment of the intact esterase with the first (N-terminal) 28 amino acids cleaved off. The cleavage of the first 28 amino acids occurs in some host cells, for example *E.Coli*. The mature peptide typically exhibits better enzymatic activity than the intact esterase.

The present invention is also directed to nucleic acids coding for the esterase, preferably the cDNA of SEQ. I.D. NO.:1 or the genomic DNA of SEQ. I.D. No.:3.

Brief Description of the Drawings:

Figure 1 Optimum temperature of the cephalosporin esterase.

Figure 2 The thermal stability of the cephalosporin esterase.

Figure 3 The pH optimum of the cephalosporin esterase.

Figure 4 The N-terminus of the protein (SEQ. I.D. NO.:9), the reverse translation sequence of the genomic N-terminus (SEQ. ID. NO.: 10), the inverse translation sequence that is complementary to the reverse translation sequence (SEQ. ID. NO.: 11), and the four oligonucleotide probes (Probes 1-4, SEQ. I.D. NOS.: 5-8, respectively) used to identify the gene for the esterase. X represents any

Figure 5a The cDNA sequence coding for the esterase of the invention (SEQ. I.D. NO.:1) and the corresponding amino acid sequence of the esterase of the invention (SEQ. I.D. NO.:2).

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Figure 5b Continuation of Figure 5a.

Figure 6a The genomic DNA sequence coding for the esterase of the invention (SEQ. I.D. NO.:3) and the corresponding amino acid sequence of the esterase of the invention (SEQ. I.D. NO.:2).

Figure 6b Continuation of Figure 6a.

Figure 7 The amino acid sequence of the esterase of the invention containing 579 amino acids (SEQ. ID. NO.: 2) showing the 551 amino acid sequence of the mature peptide(SEQ. ID. NO.: 4) which typically has better enzymatic activity than the entire protein.

Figure 8 Analysis of the amino acid composition of the intact esterase of the invention.

Detailed Description of the Invention

The present invention concerns an isolated nucleic acid molecule comprising a nucleic acid sequence coding for all or part of cephalosporin esterase from *Rhodosporidium toruloides*. A preferred strain of *Rhodospordium toruloides* is ATCC 10657 which is well known in the art and is deposited with and available from the American Type Culture Collection, Rockville, MD. and is described in U.S. patent no. 4,533,632. Preferably, the nucleic acid molecule is a DNA molecule and the nucleic acid sequence is a DNA sequence. All DNA sequences are represented herein by formulas whose left to right orientation is in the conventional direction of 5' to 3'. Nucleotide base abbreviations used herein are conventional in the art, i.e., T is thymine, A is adenine, C is cytosine, and G is guanine; also, X is A,T,C, or G, Pu is purine (i.e., G or A), and Py is pyrimidine (i.e., T or G). Further preferred is a DNA sequence having all or part of the nucleotide sequence substantially as shown in Figures 5 and 6; or a DNA sequence complementary to one of these DNA sequences; or a DNA sequence which hybridizes to a DNA sequence complementary to one of these DNA sequences. Preferably, the DNA sequence hybridizes under stringent conditions. Stringent hybridization conditions select for DNA sequences of greater than 80% homology, preferably greater than 85% or, more

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preferably, greater than 90% homology. Screening DNA under stringent conditions may be carried out according to the method described in Nature, 313: 402-404 (1985). The DNA sequences capable of hybridizing under stringent conditions with the DNA disclosed in the present application may be, for example, allelic variants of the disclosed DNA sequences, may be naturally present in *Rhodospordium toluloides* but related to the disclosed DNA sequences, or may be derived from other bacterial sources. General techniques of nucleic acid hybridization are disclosed by Maniatis, T. et al., In: Molecular Cloning, a Laboratory Manual, Cold Spring Harbor, N.Y. (19820, and by Haymes, B.D. et al., In: Nucleic Acid Hybridization, a Practical Approach, IRL Press, Washington, D.C. (1985), which references are incorporated herein by reference. In the case of a nucleotide sequence (e.g., a DNA sequence) coding for part of cephalosporin esterase, it is preferred that the nucleotide sequence be at least about 20 nucleotides in length.

Preferred DNA fragments are the probes of SEQ. ID. NOS.:5-8.

The cephalosporin esterase molecules of the present invention do not necessarily need to be catalytically active. For example, catalytically inactive cephalosporin esterase or fragments thereof may be useful in raising antibodies to the protein.

It is also contemplated that the present invention encompasses modified sequences. As used in the present application, the term "modified", when referring to a nucleotide or polypeptide sequence, means a nucleotide or polypeptide sequence which differs from the wild-type sequence found in nature.

The DNA sequences of the present invention can be obtained using various methods well-known to those of ordinary skill in the art. At least three alternative principal methods may be employed:

(i) the isolation of a double-stranded DNA sequence from genomic DNA or complementary DNA (cDNA) which contains the sequence;

(2) the chemical synthesis of the DNA sequence; and

(3) the synthesis of the DNA sequence by polymerase chain reaction (PCR).

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In the first approach, a genomic or cDNA library can be screened in order to identify a DNA sequence coding for all or part of cephalosporin esterase. For example, a *R. toruloides* genomic DNA library can be screened in order to identify the DNA sequence coding for all or part of cephalosporin esterase. Various techniques can be used to screen the genomic DNA or cDNA libraries.

For example, labeled single stranded DNA probe sequences duplicating a sequence present in the target genomic DNA or cDNA coding for all or part of cephalosporin esterase can be employed in DNA/DNA hybridization procedures carried out on cloned copies of the genomic DNA or cDNA which have been denatured to single stranded form.

A genomic DNA or cDNA library can also be screened for a genomic DNA or cDNA coding for all or part of cephalosporin esterase using immunoblotting techniques.

In one typical screening method suitable for either immunoblotting or hybridization techniques, the genomic DNA library, which is usually contained in a vector, or cDNA library is first spread out on agar plates, and then the clones are transferred to filter membranes, for example, nitrocellulose membranes. A DNA probe can then be hybridized or an antibody can then be bound to the clones to identify those clones containing the genomic DNA or cDNA coding for all or part of cephalosporin esterase.

In the second approach, the DNA sequences of the present invention coding for all or part of cephalosporin esterase can be chemically synthesized. For example, the DNA sequence coding for cephalosporin esterase can be synthesized as a series of 100 base oligonucleotides that can be sequentially ligated (via appropriate terminal restriction sites or complementary terminal sequences) so as to form the correct linear sequence of nucleotides.

In the third approach, the DNA sequences of the present invention coding for all or part of cephalosporin esterase can be synthesized using PCR. Briefly, pairs of synthetic DNA oligonucleotides at least 15 bases in length (PCR primers) that hybridize to opposite strands of the target DNA sequence are used to enzymatically amplify the intervening region of DNA on the target sequence. Repeated cycles of heat denaturation of the

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template, annealing of the primers and extension of the 3'-termini of the annealed primers with a DNA polymerase results in amplification of the segment defined by the 5' ends of the PCR primers. See, White et al., Trends Genet. 5, 185-189 (1989).

The DNA sequences of the present invention can be used in a variety of ways in accordance with the present invention. The most apparent use of the DNA sequence is to prepare cephalosporin esterase to be useful for the hydrolysis of the 3' acetyl groups of cephalosporins. However, they also can be used as DNA probes to screen other cDNA and genomic DNA libraries as to select by hybridization other DNA sequences that code for proteins related to cephalosporin esterase. In addition, the DNA sequences of the present invention coding for all or part of cephalosporin esterase can be used as DNA probes to screen other cDNA and genomic DNA libraries to select by hybridization DNA sequences that code for cephalosporin esterase molecules from organisms other than *R. toruloides.*

The DNA sequences of the present invention coding for all or part of cephalosporin esterase can also be modified (i.e., mutated) to prepare various mutations. Such mutations may be either degenerate, i.e., the mutation changes the amino acid sequence encoded by the mutated codon, or non-degenerate, i.e., the mutation does not change the amino acid sequence encoded by the mutated codon. These modified DNA sequences may be prepared, for example, by mutating the cephalosporin esterase DNA sequence so that the mutation results in the deletion, substitution, insertion, inversion or addition of one or more amino acids in the encoded polypeptide using various methods known in the art. For example, the methods of site-directed mutagenesis described in Morinaga et al., Bio/Technol. 2, 636-639 (1984), Taylor et al., Nucl. Acids Res. 13, 8749-8764 (1985) and Kunkel, Proc. Natl. Acad. Sci. USA 82, 482-492 (1985) may be employed. In addition, kits for site-directed mutagenesis may be purchased from commercial vendors. For example, a kit for performing site-directed mutagenesis may be purchased from Amersham Corp. (Arlington Heights, IL). In addition, disruption, deletion and truncation methods as described in Sayers et al., Nucl. Acids Res. 16, 791-802 (1988) may also be employed. Both degenerate and non-degenerate mutations may be advantageous in

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producing or using the polypeptides of the present invention. For example, these mutations may permit higher levels of production, easier purification, or provide additional restriction endonuclease recognition sites. All such modified DNA and polypeptide molecules are included within the scope of the present invention.

The present invention further concerns expression vectors comprising a DNA sequence coding for all or part of cephalosporin esterase. The expression vectors preferably contain all or part of one of the DNA sequences having the nucleotide sequences substantially as shown in Figures 6 or 7. Further preferred are expression vectors comprising one or more regulatory DNA sequences operatively linked to the DNA sequence coding for all or part of cephalosporin esterase. As used in this context, the term "operatively linked" means that the regulatory DNA sequences are capable of directing the replication and/or the expression of the DNA sequence coding for all or part of cephalosporin esterase.

Expression vectors of utility in the present invention are often in the form of "plasmids", which refer to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Expression vectors useful in the present invention typically contain an origin of replication, a promoter located in front (i.e., upstream of) the DNA sequence and followed by the DNA sequence coding for all or part of the structural protein. The DNA sequence coding for all or part of the structural protein is followed by transcription termination sequences and the remaining vector. The expression vectors may also include other DNA sequences known the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression product, sequences which allow expression of the structural gene to modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium), marking sequences which are capable of providing phenotypic selection in transformed host cells, stability elements such as centromeres which provide

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mitotic stability to the plasmid, and sequences which provide sites for cleavage by restriction endonucleases. The characteristics of the actual expression vector used must be compatible with the host cell which is to be employed. For example, when cloning in a fungal cell system, the expression vector should contains promoters isolated from the genome of fungal cells (e.g., the cephalosporin esterase promoter from R. *toruloides* or the *trp*C promoter from *Aspergillus nidulans*). Certain expression vectors may contain a fungal autonomously replicating sequence (ARS; e.g., ARS from *Fusarium oxysporum* and *Saccharomyces cerevisiae*) which promotes *in vivo* production of self-replicating plasmids in fungal hosts. It is preferred that the fungal expression vectors of the invention do not have a fungal ARS sequence and thus will integrate into host chromosomes upon plasmid entry of host cells. Such integration is preferred because of enhanced genetic stability. An expression vector as contemplated by the present invention is at least capable of directing the replication in *Escherichia coli* and integration in fungal cells, and preferably the expression, of the cephalosporin esterase DNA sequences of the present invention. Suitable origins of replication in *E. coli* various hosts include, for example, a ColEI plasmid replication origin. Suitable promoters include, for example, the *trp*C promoter from *A. nidulans* and the *neo*-r gene promoter from *E. coli*. Suitable termination sequences include, for example, the *trp*C terminator from *A. nidulans*, and the neo-r gene terminator from *E. coli*. It is also preferred that the expression vector include a sequence coding for a selectable marker. The selectable marker is preferably antibiotic resistance. As selectable markers, phleomycin resistance (for fungal cells), ampicillin resistance, and neomycin resistance (for bacterial cells) can be conveniently employed. All of these materials are known in the art and are commercially available.

Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

The present invention additionally concerns host cells containing an expression vector which comprises a DNA sequence coding for all or part

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of cephalosporin esterase. The host cells preferably contain an expression vector which comprises all or part of one of the DNA sequence having the nucleotide sequences substantially as shown in Figures 6 or 7. Further preferred are host cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of and operatively linked to a DNA sequence coding for all or part of cephalosporin esterase. Additionally included are host cells containing an expression vector which comprises a DNA sequence which has been modified (e.g., disrupted, deleted or truncated) so as to code for a cephalosporin esterase molecule which is not catalytically active. Suitable host cells include both eukaryotic and prokaryotic host cells, for example, *E. coli* cells. Suitable eukaryotic host cells include, for example, *R. toruloides*, *Cephalosporium acremonium*, and *Penicillium chrysogenum* cells.

Expression vectors may be introduced into host cells by various methods known in the art. For example, transfection of host cells with expression vectors can be carried out by the polyethylene glycol mediated protoplast transformation method. However, other methods for introducing expression vectors into host cells, for example, electroporation, biolistic injection, or protoplast fusion, can also be employed.

Once an expression vector has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of large amounts of the desired polypeptide, in the preferred case a polypeptide molecule comprising all or part of cephalosporin esterase.

Host cells containing an expression vector which contains a DNA sequence coding for all or part of cephalosporin esterase may be identified by one or more of the following six general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of marker gene functions; (d) assessing the level of transcription as measured by the production of cephalosporin esterase mRNA transcripts in the host cell; (d) detection of the gene product immunologically; (e) colorimetric detection; and (f) enzyme assay, enzyme assay being the preferred method of identification.

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In the first approach, the presence of a DNA sequence coding for all or part of cephalosporin esterase can be detected by DNA-DNA or RNA-DNA hybridization using probes complementary to the DNA sequence.

In the second approach, the recombinant expression vector host system can be identified and selected based upon the presence or absence of certain marker gene functions (e.g., acetamide utilization, resistance to antibiotics, resistance to fungicide, uracil prototrophy, etc.). A marker gene can be placed in the same plasmid as the DNA sequence coding for all or part of cephalosporin esterase under the regulation of the same or a different promoter used to regulate the cephalosporin esterase coding sequence. Expression of the marker gene in response to induction or selection indicates the presence of the entire recombinant expression vector which carries the DNA sequence coding for all or part of cephalosporin esterase.

In the third approach, the production of cephalosporin esterase mRNA transcripts can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by Northern blotting or nuclease protection assay using a probe complementary to the RNA sequence. Alternatively, the total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of all or part of cephalosporin esterase can be assessed immunologically, for example, by Western blotting.

In the fifth approach, the expression of cephalosporin esterase protein can be assessed by complementation analysis. For example, in cells known to be deficient in this enzyme, expression of cephalosporin esterase activity can be detected on the enzymatic hydrolysis of a colorless substrate, *p*-nitrophenylacetate, to a yellow colored *p*-nitrophenylate on the media plate.

In the sixth approach, expression of cephalosporin esterase can be measured by assaying for cephalosporin esterase enzyme activity using known methods. For example, the assay described in the Examples section hereof may be employed.

The DNA sequences of expression vectors, plasmids or DNA molecules of the present invention may be determined by various methods

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known in the art. For example, the dideoxy chain termination method as described in Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977), or the Maxam-Gilbert method as described in Proc. Natl. Acad. Sci. USA 74, 560-564 (1977) may be employed.

It should, of course, be understood that not all expression vectors and DNA regulatory sequences will function equally well to express the DNA sequences of the present invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art may make a selection among expression vectors, DNA regulatory sequences, and host cells using the guidance provided herein without undue experimentation and without departing from the scope of the present invention.

The present invention further concerns a method for producing cephalosporin esterase comprising culturing a host cell containing an expression vector capable of expressing cephalosporin esterase.

The present invention further concerns polypeptide molecules comprising all or part of cephalosporin esterase, said polypeptide molecules preferably having all or part of one of the amino acid sequence substantially as shown in Figure 5. In the case of polypeptide molecules comprising part of cephalosporin esterase, it is preferred that polypeptide molecules be at least about 10 amino acids in length.

All amino acid residues identified herein are in the natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem. 243, 3557-3559 (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

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TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	L-tyrosine
G	Gly	L-glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

All amino acid sequences are represented herein by formulas whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

The polypeptides of the present invention may be obtained by synthetic means, i.e., chemical synthesis of the polypeptide from its component amino acids, by methods known to those of ordinary skill in the art. For example, the solid phase procedure described in Houghton et al., Proc. Natl. Acad. Sci. 82, 5131-5135 (1985) may be employed. It is preferred that the polypeptides be obtained by production in prokaryotic or

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eukaryotic host cells expressing a DNA sequence coding for all or part of cephalosporin esterase, or by *in vitro* translation of the mRNA encoded by a DNA sequence coding for all or part of cephalosporin esterase. For example, the DNA sequence of Figure 6 or 7 may be synthesized using PCR as described above and inserted into a suitable expression vector, which in turn may be used to transform a suitable host cell. The recombinant host cell may then be cultured to produce cephalosporin esterase. Techniques for the production of polypeptides by these means are known in the art, and are described herein.

The polypeptides produced in this manner may then be isolated and purified to some degree using various protein purification techniques. For example, chromatographic procedures such as ion exchange chromatography, gel filtration chromatography and immunoaffinity chromatography may be employed.

In addition to hydrolyzing 3' acetyl groups, the polypeptides of the present invention may be used in a wide variety of other ways. For example, the polypeptides may be used to prepare in a known manner polyclonal or monoclonal antibodies capable of binding the polypeptides. These antibodies may in turn be used for the detection of the polypeptides of the present invention in a sample, for example, a cell sample, using immunoassay techniques, for example, radioimmunoassay or enzyme immunoassay. The antibodies may also be used in affinity chromatography for purifying the polypeptides of the present invention and isolating them from various sources.

The polypeptides of the present invention have been defined by means of determined DNA and deduced amino acid sequencing. Due to the degeneracy nature of the genetic code, whch results from there being more than one codon for most of the amino acid residues and stop signals, other DNA sequences which encode the same amino acid sequence as depicted in Figure 5 may be used for the production of the polypeptides of the present invention. In addition, it will be understood that allelic variations of these DNA and amino acid sequences naturally exist, or may be intentionally introduced using methods known in the art. These variations may be demonstrated by one or more amino acid differences in the overall

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sequence, or by deletions, substitutions, insertions, inversions or additions of one or more amino acids in said sequence. Such amino acid substitutions may be made, for example, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphiphatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, tyrosine. Other contemplated variations include salts and esters of the aforementioned polypeptides, as well as precursors of the aforementioned polypeptides, for example, precursors having N-terminal substituents such as methionine, N-formylmethionine used and leader sequences. All such variations are included within the scope of the present invention.

The following examples are further illustrative of the present invention. These examples are not intended to limit the scope of the present invention, and provide further understanding of the invention.

In the following examples, some reagents, plasmids, restriction enzymes and other materials were obtained from commercial sources and used according to the indication by suppliers. Operations employed for the purification and characterization and the cloning of DNA and the like are well known in the art or can be adapted from the literature.

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Example 1

Purification of C phalosporin Esterase

1.1 Culture of Microorganism

Rhodosporidium toruloides (ATCC 10657) seed culture was initiated from the inoculation of frozen preservation cultures of 2% into 500 ml Erlenmeyer flasks containing 100 ml of the following medium: 2% glucose, 1% yeast extract, 1% Bacto-peptone, 0.5% KH_2PO_4, pH 6.0. Seed flasks were cultured for 24 hours at 28°C and 250 rpm; 2% inoculum volume was used to start production stage fermentations. Production stage medium was composed of: 8% corn steep liquor, 1% KH_2PO_4, 3% glucose, pH 6.2. The media was autoclaved for two hours. This led to increased titers when compared to the normal autoclave time of 30 minutes. Fermentor broth was cultured for 3 or 4 days to 16-21°C with high aeration. Specific activities of whole broth were typically in the range of 20-37 IU/ml.

1.2 Purification of the Enzyme From *Rhodosporidium Toruloides*

The esterase was released from *Rhodosporidium toruloides* cells by treatment of the fermentation broth with 100 mM EDTA at pH 4.0 for 8 hours. Approximately 50% of the enzymatic activity could be released from the cells in this manner. The broth was centrifuged at 5000 g to remove the cells and the corn steep solids. The supernatant was ultrafiltered through an Amicon hollow fiber cartridge with a molecular weight cut-off of 30,000 to 10% of the original volume. The enzyme was brought up to the original volume by addition of deionized water. The pH was brought up to 7.0 by addition of 2 M ammonium hydroxide and the enzyme solution added to DEAE Trisacryl (100 g resin/50 ml enzyme solution) which had been washed with 50 mM potassium phosphate buffer 7.0. The enzyme does not bind to DEAE and was obtained in the filtrate which was then brought to pH 4.5 with 1.0 M acetic acid. This solution was then loaded onto a carboxymethyl Sepharose column (18 x 3 cm) and washed with 50 mM ammonium acetate pH 4.5 until the absorbance at 280 nm was less than 0.1 (approximately 4

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column volumes). The esterase was eluted with a linear gradient of 50 to 500 mM ammonium acetate pH 6.5 (flow rate 1.0 ml/min). Fractions of 7.0 ml were collected and the fractions containing esterase were pooled and concentrated on a 50,000 molecular weight cut off Centricon.

Example 2

Characterization of Cephalosporin Esterase

2.1 Specific Activity of Enzyme

Enzyme was added to the reaction mixture containing the potassium salt of the cephalosporin (25-400 mM), 100 mM potassium phosphate, pH 6.5 in a final volume of 0.5 ml. The mixture was incubated at 30°C (unless described otherwise) and stopped by addition of 2.0 ml 50% acetonitrile. The reaction was monitored at 254 nm by HPLC on a 5 micron C18 column (50 x 4 mm) with the mobile phase consisting of 25 mM octane sulfonic acid, 0.1% phosphoric acid, 12% methanol, pH 2.5. Protein was assayed using the Bio-Rad protein assay kit (Bio-Rad Co., USA) using bovine serum albumin as the standard. The enzyme exhibited Michaelis-Menton kinetics with cephalosporin C. From double reciprocal plots, the K_m for hydrolysis of cephalosporin C was found to be 51.8 mM with a corresponding V_{max} of 77.0 µmole/min/mg. The reaction products, desacetyl cephalosporin C and acetate did not inhibit the reaction to any appreciable extent. A 1.0% solution of cephalosporin C was completely hydrolyzed within 30 minutes at 30°C with no side products observed by HPLC.

2.2 Substrate File

Esterase activity was measured using *p*-nitrophenyl ester substrates as well as cephalosporin derivatives. The enzyme was incubated at 30°C (unless described otherwise) with *p*-nitrophenyl acetate, 10.0 mM in 100 mM potassium phosphate buffer pH 6.5 or 10.0 mM *p*-nitrophenyl esters ranging in carbon chain length from C:2 to C:18 in 100 mM potassium phosphate pH 6.5 and 2% acetonitrile. Enzyme activity was monitored

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spectrophotometrically by measuring the increase in absorbance at 405 nm due to the formation of the *p*-nitrophenylate ion. The assay for cephalosporin derivatives was as described in Example 2.1. The results are described in Table 1 for *p*-nitrophenyl ester substrates and Table 2 for cephalosporin derivatives.

TABLE 1. Effect of Increasing Ester Chain Length on Esterase Activity.

Length of Ester	**Relative Activity (%)**
Acetate C:2	100
Propionate C:3	34
Butyrate C:4	5
Caproate C:6	0
Caprylate C:8	0
Caprate C:10	0
Laurate C:12	0
Myristate C:14	0
Palmitate C:16	0
Stearate C:18	0

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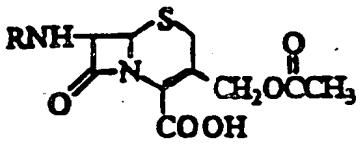
Table 2: Relative rates of esterase activity against c phem substrates



Substrate	Relative Rate
R= —H	100
—C(=O)CH$_3$	51
—C(=O)CH$_2$Cl	105
—C(=O)CHCl$_2$	108
—C(=O)CH$_2$Br	114
—C(=O)CH$_2$I	103
—C(=O)CH$_2$CH$_2$CH$_2$C(=O)OH	105
—C(=O)CH$_2$CH$_2$CH$_2$CH(NH$_2$)C(=O)OH	68
—C(=O)CH$_2$CH$_2$CH$_2$CH(NHC(=O)CH$_3$)C(=O)OH	42
—C(=O)CH$_2$-C$_6$H$_5$	17
—C(=O)CH$_2$-C$_6$H$_4$-CH$_3$	68
—C(=O)CH$_2$-C$_6$H$_4$-OCH$_3$	41
—C(=O)CH$_2$-C$_4$H$_3$S	34

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2.3 Effect of Temperature

A. Optimum Temperature

Enzyme was incubated with *p*-nitrophenyl acetate, 10.0 mM n 100 mM potassium phosphate buffer pH 6.5. The reaction mixtures were incubated for 10 minutes in a shaking water bath at 300 rpm at temperatures from 10 to 65°C. The optimal temperature for the reaction was 25°C. The results are shown in Figure 1.

B. Thermal Stability

Enzyme was incubated with *p*-nitrophenyl acetate as described in Example 2.3A. Enzyme was incubated at various temperatures for 15 minutes then immediately placed on ice. The enzyme was unstable when incubated at temperatures about 25°C with rapid inactivation between 30 and 45°C. The results are shown in Figure 2.

2.4 Effect of pH

Enzyme was incubated with *p*-nitrophenyl acetate as described in Example 2.3A. A 100 mM Tris-maleate universal buffer with a pH range of 4 to 8 was used. The esterase was found to be active in a pH range of 4.5 to 7 with optimal activity at a pH of 6.0 with both *p*-nitrophenyl acetate and cephalosporin C. The results with *p*-nitrophenyl acetate are shown in Figure 3.

2.5 Effect of Various Enzyme Modulators

Enzyme was incubated in the presence of 10 mM reagent for 15 minutes at 25°C. The reaction mixture was then diluted 100 fold into assay mix and assayed with *p*-nitrophenyl acetate. The results are summarized in Table 3. The results strongly suggests the presence of an active-site serine for the *Rhodosporidium* enzyme. Phenylmethylsulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin (DCI), and dimethyl phosphite all inhibited the enzyme. The histidine-modifying reagent diethylpyrocarbonate essentially inactivated the enzyme. Sulfhydryl-modifying agents iodoacetamide and N-ethylmaleimide had little or no effect on the activity of the enzyme although

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slight activation was observed with β-mercaptoethanol and dithiothreitol. The presence or absence of metal ions also had little or no effect on the enzyme although slight inhibition was observed with EDTA.

2.6 Determination of Isoelectric Point (pI).

Isoelectric focusing gels were run using the Ampholine PAGplate system developed by Pharmacia Biotech (Sweden) in the pH range of 3-9. pI was also determined using the MinpHor system developed by Rainin Co. (USA) with the broad range ampholyte mixture pH 3-9. The isoelectric point of the protein was determined to be approximately 5.6.

2.7 Determination of Molecular Weight

Molecular weight was determined by gel permeation chromatography and gel electrophoresis. SDS-PAGE gels (gradient 8-25%) were run according to the method of Laemmli (Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685). Proteins were stained with Coomassie brilliant blue. Gel permeation chromatography was performed by HPLC on a 75 x 300 mm TosoHaas TSK-GEL GS3000SW XL column with a mobile phase of 200 mM potassium phosphate pH 6.8, 150 mM sodium chloride. Bio-Rad gel filtration standard mixture (MW 670,000-1,350) was used as the marker. The flow rate was 1.0 ml/min and the eluate was monitored at 280 nm. Fractions were collected and assayed for esterase activity. A single band at 80,000 Da was observed by SDS-PAGE; gel filtration chromatography of the enzyme indicated that the enzyme is a monomer in the native state.

2.8 Determination of Carbohydrate Content of Enzyme

Removal of carbohydrate with recombinant peptide N-glycosidase was performed as described by Elder et al. and endoglycosidase H as performed by Trimble et al. Native and deglycosylated enzyme were then analyzed by SDS-PAGE as described in Example 2.7 to determine

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carbohydrate loss. Treatment of the enzyme with endoglycosidases resulted in a 15-20% reduction of molecular weight to approximately 62,000 Daltons.

2.9 Determination of N-Terminal Amino Acid Sequence

The amino-terminal sequence was determined by automated Edman degradation at the Cornell University Biotechnology Analytical Facility. The amino terminal sequence obtained from the purified enzyme was H_2N-Thr-Asn-Pro-Asn-Glu-Pro-Pro-Pro-Val-Val-Asp-Leu-Gly-Tyr-Ala.

3.0 Preparation of Chromosomal DNA of *R. toruloides*

Seed media culture was inoculated at 4% with a frozen culture of *Rhodosporidium toruloides* (ATCC 10657). The culture was grown at 28°C for 24 hours in 2% glucose, 1% yeast extract, 1% bacto-peptone, 0.5% KH_2PO_4, pH 6.0. Cells were harvested by centrifugation and washed once in buffer containing: 1M sorbitol, 50 mM sodium citrate pH 5.4. Cells were centrifuged again and resuspended in wash buffer containing 0.5% lysing enzymes (Sigma Chemical Co., USA) at 37°C for 3 hours. Spheroplasts were collected by centrifugation and digested in 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 01% SDS and 100 µg/ml proteinase K. The solution was incubated at 50°C for 16 hours. The mixture was extracted twice, first with phenol:chloroform:isoamyl alcohol (24:24:1), then with chloroform:isoamylalcohol (24:1) and the DNA was precipitated with ethanol (70%). The DNA was recovered by centrifugation and washed with 70% ethanol. The DNA pellet was dissolved in TE (10 mM Tris-HCl pH 8.0, 1mM EDTA) and 100 µg/ml Rnase A and incubated for at 37°C for 16 hours. The organic extractions and ethanol precipitations were repeated and the DNA was dissolved in TE. The DNA concentration was determined spectrophotometrically.

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3.1 Construction of Genomic DNA Library of *R. Toruloides*

From the N-terminal amino acid sequence (section 2.9) four 17-omer oligonucleotide probes were synthesized (Figure 4), end-labeled with [γ-32P]ATP, and used to probe a southern blot of R. toruloides chromsomal DNA digested with restriction endonucleases BamH1 and Pst1. Hybridization was conducted in TMAC (tetramethylammoniumchloride, Sigma chemical Co.) buffer at 46.8°C for 48 hours. A 3 kb BamH1 fragment hybridized to one of the probes. The 3 kb BamH1 fragment was isolated and ligated to pBluescript KS+ phagemid (Stratagene, USA) cleaved with BamH1 and treated with bacterial alkaline phosphatase. The ligation mixture was used to transform E. coli XL1-blue cells by electroporation at 2.5 kvolts, 200 ohms, 25μFd. The transformants were selected onLB agar containing 100 μg/ml ampicillin.

3.2 Selection of Clone Containing Cephalosporin Esterase Gene

Colony blots of the genomic library were prepared and screened with the N-terminal oligonucleotide probe. Twelve clones were initially selected for further evaluation. Plasmid DNA was isolated from each transformant using the TELT mini-prep method (He et al. 1990 Nucl. Acid Res., 18:1660). Southern analysis of these clones identified two that hybridized to the probe. Translation of the adjacent DNA sequence produced an amino acid sequence that was identical to the N-terminal protein sequence. Further analysis of the 3 kb BamH1 fragment by primer extension and southern blotting determined the location and orientation of the esterase gene within the fragment.

3.3 cDNA Cloning

A cDNA clone was produced by 3'RACE (rapid amplification of cDNA ends, BRL Co., USA). Total RNA from *R. toruloides* was isolated using Trizol reagent (BRL Co., USA) and further purified by lithium chloride precipitation. First strand cDNA was prepared by reverse transcription from an adapter primer. The RNA template was digested with Rnase H and the

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cDNA was amplified by PCR using a gene-specific primer and an adapter primer. The coding region was amplified and mutagenized by a second round of PCR using an internal gene-specific primer which included the putative translation start site and an Nco1 restriction site at the translation start site for subsequent cloning into expression vectors. This produced a 1.9 kb fragment which was gel purified. Restriction analysis and nucleotide sequencing of this fragment confirmed that it contained the esterase gene. To further facilitate cloning into an expression vector, another cDNA clone was developed that included a BspH1 site at the beginning of the mature peptide and a BamH1 site at the 3-end of the gene.

3.4 Determination of Nucleotide Sequence

The nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977 Proc. Natl. Acad. Sci. USA 74:5463-5467) using the Taq Track fmol DNA sequencing systems (Promega Co., USA). T3, T7, and synthesized internal primers were used to sequence the entire gene from both strands. Electrophoresis was performed on a 7% Long Ranger (AT Biochem. Co., USA) polyacrylamide gel containing 7M urea in TBE buffer at 2700 volts. The complete nucleotide sequence is shown in Figure 5. The coding cDNA region is 1716 bp long and codes for a 572 amino acid protein of molecular weight 61.3 kD. This is consistent with the deglycosylate form of the enzyme (Section 2.8). The N-terminal protein sequence determined from the DNA sequence is identical to the protein sequence identified in section 2.9. This sequence begins 28 residues down from the putative ATG translation start site. The cDNA clone was also sequenced for comparison to the genomic clone. The gene contains five introns which are identified in Figure 7.

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WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule having a sequence coding for the amino acid sequence of SEQ. ID. NOS: 2 or 4.

2. An isolated nucleic acid molecule having a sequence complementary to a nucleic acid sequence coding for the amino acid sequence of SEQ. ID. NOS: 2 or 4.

3. An isolated nucleic acid molecule having a sequence capable of hybridizing under stringent conditions to a nucleic acid having a sequence complementary to a nucleic acid sequence coding for the amino acid sequence of SEQ. ID. NOS: 2 or 4.

4. The nucleic acid molecule of Claim 1 which is a DNA molecule.

5. The nucleic acid molecule of Claim 2 which is a DNA molecule.

6. The nucleic acid molecule of Claim 3 which is a DNA molecule

7. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.:1.

8. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.:3.

9. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.: 5.

10. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.: 6.

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11. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.: 7.

12. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.:8.

13. An isolated polypeptide having the amino acid sequence of SEQ. ID. NO.:2.

14. An isolated polypeptide having the amino acid sequence of SEQ. ID. NO.:4.

15. An expression vector comprising

a. a nucleic acid molecule having a sequence coding for the amino acid sequence of SEQ. ID. NOS.: 2 or 4;

b. a nucleic acid molecule having a sequence complementary to a nucleic acid molecule having a sequence coding for the amino acid sequence of SEQ. ID. NOS: 2 or 4; or

c. a nucleic acid molecule having a sequence capable of hybridizing under stringent conditions to a nucleic acid molecule having a sequence complementary to a nucleic acid molecule having a sequence coding for the amino acid sequence of SEQ. ID. NOS.: 2 or 4.

16. The expression vector of Claim 15 further comprising an origin of replication, a promoter, and a transcription termination sequence.

17. The expression vector of Claim 16 further comprising a selectable marker sequence.

18. The expression vector of Claim 16 which is capable of integrating into fungal chromosomes.

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19. The expression vector of Claim 15 having the DNA sequence of SEQ. ID. NOS.:1 or 3.

20. The expression vector of Claim 15 which is a plasmid.

21. A host cell containing the expression vector of Claim 15.

22. A host cell containing the expression vector of Claim 18.

23. A host cell containing the expression vector of Claim 19.

24. The host cell of Claim 21 which is eukaryotic.

25. The host cell of Claim 22 which is eukaryotic.

26. A method for producing a polypeptide having cephalosporin esterase activity comprising culturing the host cell of Claim 21 under conditions resulting in expression of the polypeptide.

27. The host cell of Claim 21 selected from the group consisting of the species *Escherichia coli, Rhodospordium toruloides, Cephalosporin acremonium,* and *Penicilluim chrysagenum.*

28. The host cell of Claim 21 which is the species *Cephalosporin acremonium.*

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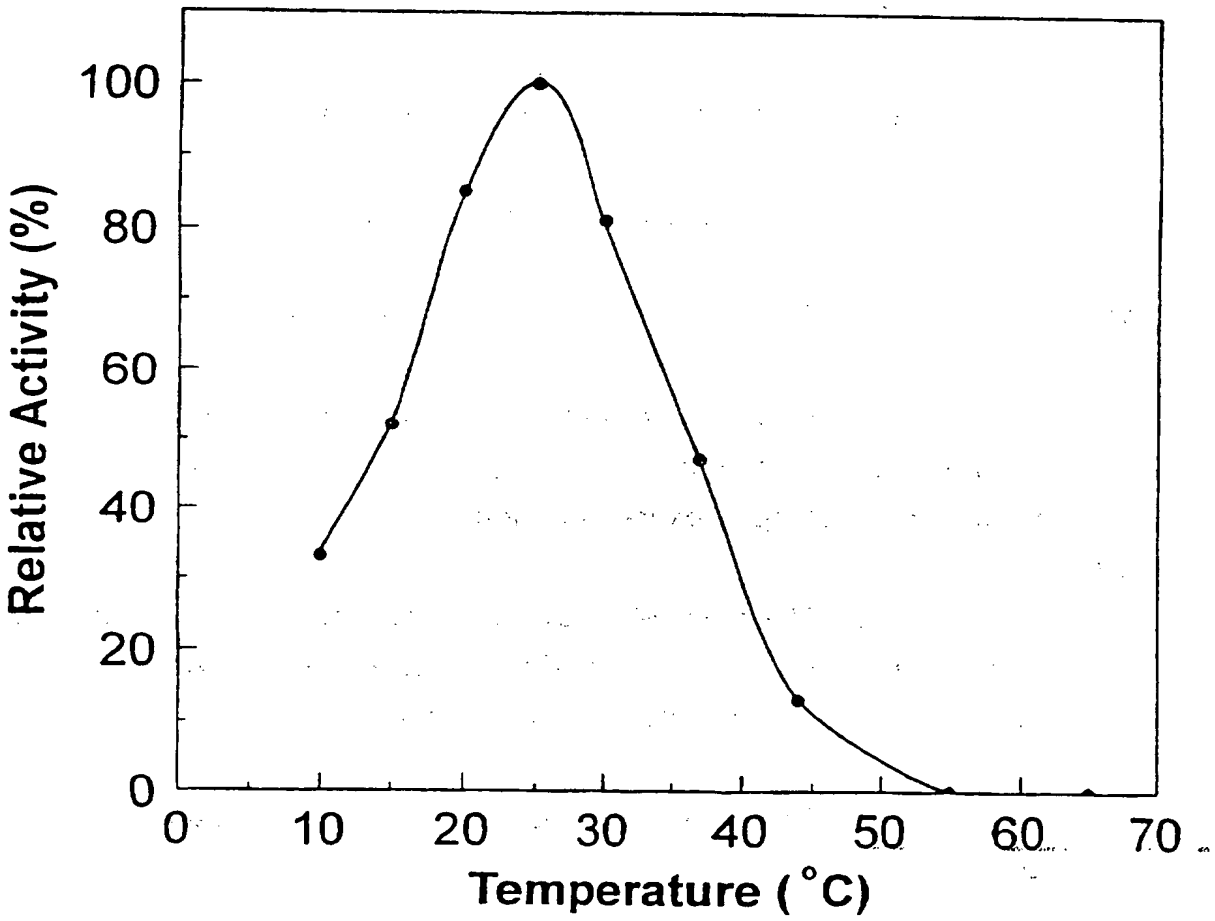
Relative Activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temperature (°C)

FIG. 1

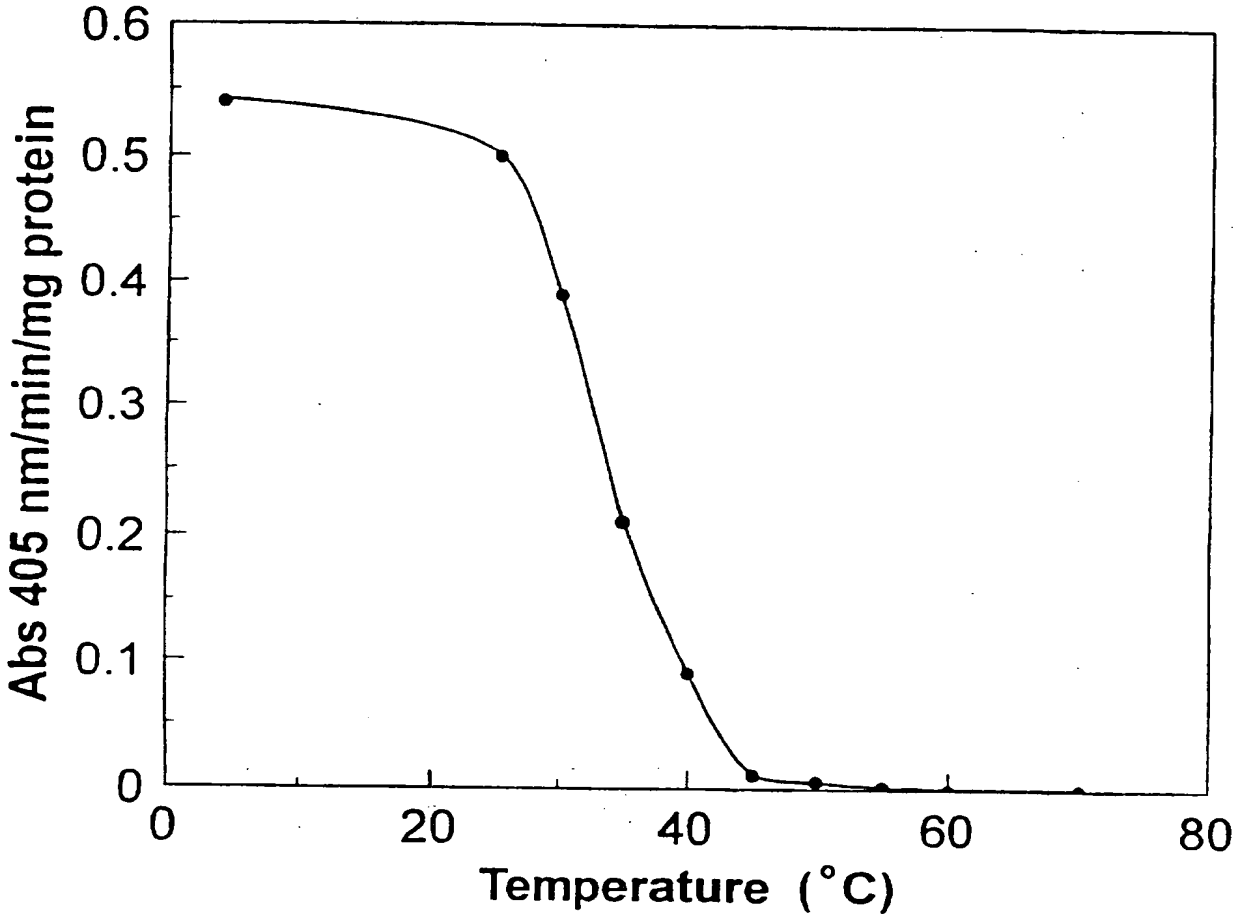
0.6
0.5
0.4
0.3
0.2
0.1
0
Abs 405 nm/min/mg protein
0
20
40
60
80
Temperature (°C)

FIG. 2

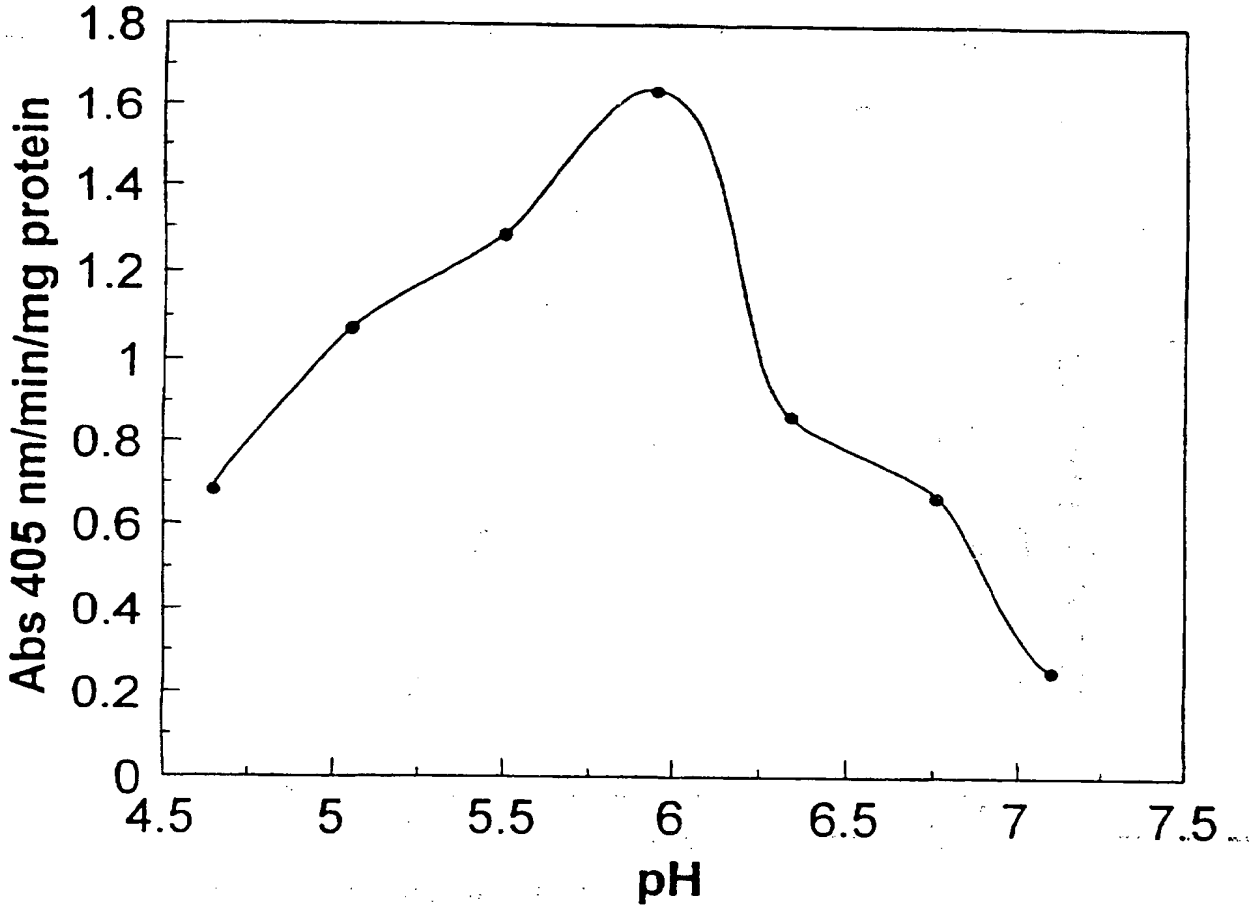
Abs 405 nm/min/mg protein
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
4.5
5
5.5
6
6.5
7
7.5
pH

FIG. 3

AMINO ACID SEQ.		T	N	P	N	E	P
REV. TRANSLATION		ACX	AAPy	CCX	AAPy	GAPu	CC
INVERSE		GGPy	TCPu	TTX	GGPu	TTX	GT
PROBE	1	GGPy	TCPu	TTG	GGPu	TTX	GT
	2			A			
	3			T			
	4			C			

Four 17-mer oligonucleotide probes each with a 32-fold degeneracy were synthesized from the N-terminal amino acid sequence and used to probe a Southern blot of *R.toruloides* DNA

FIG. 4

BNSDOCID: <WO__9812345A1_I_>

```
ACTCGCCGCCATGCTCCTTAACCTCTTCACCCTCGCCTCCCTCGCTGCGACGCTCCAGCT      60
 L  A  A  M  L  L  N  L  F  T  L  A  S  L  A  A  T  L  Q  L

CGCCTTTGCCTCTCCGACCTCCCTCGTCCGCCGCACGAACCCAAACGAGCCCCCTCCCGT     120
 A  F  A  S  P  T  S  L  V  R  R  T  N  P  N  E  P  P  P  V

CGTCGACCTCGGCTACGCCCGCTACCAAGGCTACTTGAACGAGACCGCCGGACTCTACTG     180
 V  D  L  G  Y  A  R  Y  Q  G  Y  L  N  E  T  A  G  L  Y  W

GTGGCGCGGAATCCGCTACGCCTCGGCTCAGCGCTTCCAGGCTCCTCAGACGCCCGCGAC     240
 W  R  G  I  R  Y  A  S  A  Q  R  F  Q  A  P  Q  T  P  A  T

GCACAAGGCCGTCCGCAACGCGACTGAGTATGGACCGATCTGTTGGCCGGCTAGCGAGGG     300
 H  K  A  V  R  N  A  T  E  Y  G  P  I  C  W  P  A  S  E  G

AACCAACACGACCAAGGGCTTGCCGCCGCCTAGCAACAGCTCGAGCAGCGCGCCGCAGAA     360
 T  N  T  T  K  G  L  P  P  P  S  N  S  S  S  S  A  P  Q  K

ACAGGCGTCGGAGGATTGCCTCTTCCTCAATGTCGTTGCCCCCGCCGGCTCGTGCGAGGG     420
 Q  A  S  E  D  C  L  F  L  N  V  V  A  P  A  G  S  C  E  G

CGACAATCTTCCCGTCCTCGTCTACATTCACGGAGGTGGCTACGCCTTCGGCGATGCGAG     480
 D  N  L  P  V  L  V  Y  I  H  G  G  G  Y  A  F  G  D  A  S

CACCGGCAGCGACTTTGCCGCCTTCACCAAGCACACGGGAACCAAGATGGTCGTTGTAAA     540
 T  G  S  D  F  A  A  F  T  K  H  T  G  T  K  M  V  V  V  N

TCTCCAGTACCGTCTCGGCAGCTTTGGTTTCCTCGCTGGCCAAGCCATGAAGGACTACGG     600
 L  Q  Y  R  L  G  S  F  G  F  L  A  G  Q  A  M  K  D  Y  G

TGTAACGAACGCCGGCTTGCTTGACCAGCAATTCGCCCTTCAATGGGTTCAACAGCACGT     660
 V  T  N  A  G  L  L  D  Q  Q  F  A  L  Q  W  V  Q  Q  H  V

CTCGAAGTTCGGCGGCAACCCCGATCACGTTACGATTTGGGGCGAGTCTGCAGGCGCAGG     720
 S  K  F  G  G  N  P  D  H  V  T  I  W  G  E  S  A  G  A  G

GTCCGTTATGAACCAGATCATTGCGAACGGCGGCAACACCGTCAAGGCTCTCGGTCTCAA     780
 S  V  M  N  Q  I  I  A  N  G  G  N  T  V  K  A  L  G  L  K

GAAGCCCCTCTTCCACGCTGCCATCGGCTCCTCCGTCTTCCTCCCCTACCAAGCCAAGTA     840
 K  P  L  F  H  A  A  I  G  S  S  V  F  L  P  Y  Q  A  K  Y

CAACTCCCCCTTCGCCGAGCTGCTCTACTCCCAACTCGTCTCGGCGACAAACTGCACCAA     900
 N  S  P  F  A  E  L  L  Y  S  Q  L  V  S  A  T  N  C  T  K

AGCCGCCTCGTCCTTCGCTTGCCTCGAAGCTGTCGACGCTGCGGCGCTCGCTGCGGCGGG     960
 A  A  S  S  F  A  C  L  E  A  V  D  A  A  A  L  A  A  A  G

CGTGAAGAACTCGGCGGCGTTCCCGTTCGGGTTTTGGTCGTATGTCCCGGTCGTCGACGG    1020
 V  K  N  S  A  A  F  P  F  G  F  W  S  Y  V  P  V  V  D  G

GACCTTCTTGACTGAGCGCGCGTCGCTCCTTCTCGCCAAGGGCAAGAAGAACCTCAATGG    1080
 T  F  L  T  E  R  A  S  L  L  L  A  K  G  K  K  N  L  N  G

CAACCTCTTCACCGGGATCAACAACCTCGACGAAGGATTCATATTCACTGACGCCACTAT    1140
```

FIG. 5A

```
 N  L  F  T  G  I  N  N  L  D  E  G  F  I  F  T  D  A  T  I

TCAGAACGACACGATCAGCGACCAGTCGCAGCGCGTCTCCCAGTTCGACCGCCTCCTCGC      1200
 Q  N  D  T  I  S  D  Q  S  Q  R  V  S  Q  F  D  R  L  L  A

CGGCCTCTTCCCCTACATCACCTCGGAGGAGCGCCAGGCCGTCGCGAAGCAGTACCCGAT      1260
 G  L  F  P  Y  I  T  S  E  E  R  Q  A  V  A  K  Q  Y  P  I

CTCCGACGCGCCGTCAAAGGGCAACACCTTCTCTCGCATCTCGGCCGTCATCGCGGACTC      1320
 S  D  A  P  S  K  G  N  T  F  S  R  I  S  A  V  I  A  D  S

GACCTTCGTCTGCCCGACCTACTGGACCGCCGAGGCGTTCGGCTCGTCCGCCCACAAGGG      1380
 T  F  V  C  P  T  Y  W  T  A  E  A  F  G  S  S  A  H  K  G

CCTCTTCGACTACGCGCCGGCTCACCACGCGACCGACAACTCGTACTACATCGGCTCCAT      1440
 L  F  D  Y  A  P  A  H  H  A  T  D  N  S  Y  Y  I  G  S  I

CTGGAACGGCAAGAAGTCGGTCTCGTCCGTCCAGTCCTTCGACGGCGCGCTCGGCGGCTT      1500
 W  N  G  K  K  S  V  S  S  V  Q  S  F  D  G  A  L  G  G  F

CATCGAGACGTTCAACCCGAACAACAACGCTGCCAACAAGACCATCAACCCTTACTGGCC      1560
 I  E  T  F  N  P  N  N  N  A  A  N  K  T  I  N  P  Y  W  P

GACGTTCGACTCGGGCAAGCAGCTCCTCTTCAACACGACGACGAGGGACACCCTCTCTCC      1620
 T  F  D  S  G  K  Q  L  L  F  N  T  T  T  R  D  T  L  S  P

CGCCGACCCGCGCATCGTTGAGACTTCAAGCTTGACCGACTTTGGCACGAGCCAGAAGAC      1680
 A  D  P  R  I  V  E  T  S  S  L  T  D  F  G  T  S  Q  K  T

CAAGTGCGACTTCTGGCATGGGTCAATCTCGGTGAACGCGGGTCTCTAGGCGTCTTTC       1738
 K  C  D  F  W  H  G  S  I  S  V  N  A  G  L  *  A  S  F
```

FIG. 5B

```
GGATCCACCCGAACTCTGTCCCGCTTTCTGGCTTTCTTCCTTGCTGTCGCCCCATCGCCT        60

                |-- Translation Start -->
TTCCCGACTCGCCGCCATGCTCCTTAACCTCTTCACCCTCGCCTCCCTCGCTGCGACGCT        120
                  M  L  L  N  L  F  T  L  A  S  L  A  A  T  L

                                                |- Mature peptide ->
CCAGCTCGCCTTTGCCTCTCCGACCTCCCTCGTCCGCCGCACGAACCCAAACGAGCCCCC        180
 Q  L  A  F  A  S  P  T  S  L  V  R  R  T  N  P  N  E  P  P

TCCCGTCGTCGACCTCGGCTACGCCCGCTACCAAGGCTACTTGAACGAGACCGCCGGACT        240
 P  V  V  D  L  G  Y  A  R  Y  Q  G  Y  L  N  E  T  A  G  L

CTACTGGTGGCGCGGAATCCGCTACGCCTCGGCTCAGCGCTTCCAGGCTCCTCAGACGCC        300
 Y  W  W  R  G  I  R  Y  A  S  A  Q  R  F  Q  A  P  Q  T  P

CGCGACGCACAAGGCCGTCCGCAACGCGACTGAGTATGGACCGATCTGTTGGCCGGCTAG        360
 A  T  H  K  A  V  R  N  A  T  E  Y  G  P  I  C  W  P  A  S

CGAGGGAACCAACACGACCAAGGGCTTGCCGCCGCCTAGCAACAGCTCGAGCAGCGCGCC        420
 E  G  T  N  T  T  K  G  L  P  P  P  S  N  S  S  S  S  A  P

GCAGAAACAGGCGTCGGAGGATTGCCTCTTCCTCAATGTCGTTGCCCCCGCCGGCTCGTG        480
 Q  K  Q  A  S  E  D  C  L  F  L  N  V  V  A  P  A  G  S  C

CGAGGGCGACAATCTTCCCGTCCTCGTCTACATTCACGGAGGTGGCTACGCCTTCGGCGA        540
 E  G  D  N  L  P  V  L  V  Y  I  H  G  G  G  Y  A  F  G  D

TGCGAGCACCGGCAGCGACTTTGCCGCCTTCACCAAGCACACGGGAACCAAGATGGTCGT        600
 A  S  T  G  S  D  F  A  A  F  T  K  H  T  G  T  K  M  V  V

TGTAAATCTCCAGTACCGTCTCGGCAGCTTTGGTTTCCTCGCTGGCCAAGCCATGAAGGA        660
 V  N  L  Q  Y  R  L  G  S  F  G  F  L  A  G  Q  A  M  K  D

                                        [---- Intron #1 ----------
CTACGGTGTAACGAACGCCGGCTTGCTTGACCAGGTGAGTTTCCCGCATGATACCCGCCC        720
 Y  G  V  T  N  A  G  L  L  D  Q

-------------------------------------]
ACCTTTCGACTCATGCTGACGCCTCTCCCGCTCGCAGCAATTCGCCCTTCAATGGGTTCA        780
                                      Q  F  A  L  Q  W  V  Q

ACAGCACGTCTCGAAGTTCGGCGGCAACCCCGATCACGTTACGATTTGGGGCGAGTCTGC        840
 Q  H  V  S  K  F  G  G  N  P  D  H  V  T  I  W  G  E  S  A

                                      [---- Intron #2 -------
AGGCGCAGGGTCCGTTATGAACCAGATCATTGCGAACGTGAGCCACCCGAACCGATCTCC        900
 G  A  G  S  V  M  N  Q  I  I  A  N

-----------------------------------------------------]
AGCCGACTTTCCCCCCCCCCCCCCCCCCCGCTGACCTCCCTCGTCTTGCAGGGCGGCAACA        960
                                                    G  G  N  T

CCGTCAAGGCTCTCGGTCTCAAGAAGCCCCTCTTCCACGCTGCCATCGGCTCCTCCGTCT       1020
 V  K  A  L  G  L  K  K  P  L  F  H  A  A  I  G  S  S  V  F

TCCTCCCCTACCAAGCCAAGTACAACTCCCCCTTCGCCGAGCTGCTCTACTCCCAACTCG       1080
 L  P  Y  Q  A  K  Y  N  S  P  F  A  E  L  L  Y  S  Q  L  V
```

FIG. 6A

BNSDOCID: <WO__ 9812345A1 I >

```
TCTCGGCGACAAACTGCACCAAAGCCGCCTCGTCCTTCGCTTGCCTCGAAGCTGTCGACG      1140
  S  A  T  N  C  T  K  A  A  S  S  F  A  C  L  E  A  V  D  A

CTGCGGCGCTCGCTGCGGCGGGCGTGAAGAACTCGGCGGCGTTCCCGTTCGGGTTTTGGT      1200
  A  A  L  A  A  A  G  V  K  N  S  A  A  F  P  F  G  F  W  S

CGTATGTCCCGGTCGTCGACGGGACCTTCTTGACTGAGCGCGCGTCGCTCCTTCTCGCCA      1260
  Y  V  P  V  V  D  G  T  F  L  T  E  R  A  S  L  L  L  A  K

                                [---- Intron #3 ----------------------
AGGGCAAGAAGAACCTCAATGGCGTGCGTGGCGAGCTTTCGAGTGCTTCAGGATCTCGCT      1320
  G  K  K  N  L  N  G

----------------------]                                        [---
GACACTGTCGACCGGCTCGCAGAACCTCTTCACCGGGATCAACAACCTCGACGAAGATGA      1380
                       N  L  F  T  G  I  N  N  L  D  E  G

---- Intron #4 ----------------------------------------------]
GTTCCCGTCGACGGCTCTGTTCGCCCAGCGAGACTGACTTGTTCTTTTGCGAAGATTACG      1440

ATTCATATTCACTGACGCCACTATTCAGAACGACACGATCAGCGACCAGTCGCAGCGCGT      1500
  F  I  F  T  D  A  T  I  Q  N  D  T  I  S  D  Q  S  Q  R  V

CTCCCAGTTCGACCGCCTCCTCGCCGGCCTCTTCCCCTACATCACCTCGGAGGAGCGCCA      1560
  S  Q  F  D  R  L  L  A  G  L  F  P  Y  I  T  S  E  E  R  Q

GGCCGTCGCGAAGCAGTACCCGATCTCCGACGCGCCGTCAAAGGGCAACACCTTCTCTCG      1620
  A  V  A  K  Q  Y  P  I  S  D  A  P  S  K  G  N  T  F  S  R

                                [---- Intron #5 ------------
CATCTCGGCCGTCATCGCGGACTCGACCTTCGTGTGCGTTCCCCGTCGTCTTCTCCGAGT      1680
  I  S  A  V  I  A  D  S  T  F  V

----------------------------]
ATTCCGCTGACTTCCCGCTTGCCCGCAGCTGCCCGACCTACTGGACCGCCGAGGCGTTCG      1740
                             C  P  T  Y  W  T  A  E  A  F  G

GCTCGTCCGCCCACAAGGGCCTCTTCGACTACGCGCCGGCTCACCACGCGACCGACAACT      1800
  S  S  A  H  K  G  L  F  D  Y  A  P  A  H  H  A  T  D  N  S

CGTACTACATCGGCTCCATCTGGAACGGCAAGAAGTCGGTCTCGTCCGTCCAGTCCTTCG      1860
  Y  Y  I  G  S  I  W  N  G  K  K  S  V  S  S  V  Q  S  F  D

ACGGCGCGCTCGGCGGCTTCATCGAGACGTTCAACCCGAACAACAACGCTGCCAACAAGA      1920
  G  A  L  G  G  F  I  E  T  F  N  P  N  N  N  A  A  N  K  T

CCATCAACCCTTACTGGCCGACGTTCGACTCGGGCAAGCAGCTCCTCTTCAACACGACGA      1980
  I  N  P  Y  W  P  T  F  D  S  G  K  Q  L  L  F  N  T  T  T

CGAGGGACACCCTCTCTCCCGCCGACCCGCGCATCGTTGAGACTTCAAGCTTGACCGACT      2040
  R  D  T  L  S  P  A  D  P  R  I  V  E  T  S  S  L  T  D  F

TTGGCACGAGCCAGAAGACCAAGTGCGACTTCTGGCGTGGGTCAATCTCGGTGAACGCGG      2100
  G  T  S  Q  K  T  K  C  D  F  W  R  G  S  I  S  V  N  A  G

GTCTCTAGGCGTCTTTCCTTCCGACTTCCTTCGTTCTTTCGTTGTTTATTCTTGCAGTTC      2160
  L  *

CGTTGTATCGGCCATTCGTGCGTGTAGCTCACTCGAGTATAGACGTTGGCAAGTGCGAAA      2220
```

FIG. 6B

BNSDOCID: <WO___9812345A1_I_>

```
   |--Translation Start-->      |-Mature peptide->
LAAMLLNLFTLASLAATLQLAFASPTSLVRRTNPNEPPPVVDLGYARYQGYLNETAGLYW

WRGIRYASAQRFQAPQTPATHKAVRNATEYGPICWPASEGTNTTKGLPPPSNSSSSAPQK

QASEDCLFLNVVAPAGSCEGDNLPVLVYIHGGGYAFGDASTGSDFAAFTKHTGTKMVVVN

LQYRLGSFGFLAGQAMKDYGVTNAGLLDQQFALQWVQQHVSKFGGNPDHVTIWGESAGAG

SVMNQIIANGGNTVKALGLKKPLFHAAIGSSVFLPYQAKYNSPFAELLYSQLVSATNCTK

AASSFACLEAVDAAALAAAGVKNSAAFPFGFWSYVPVVDGTFLTERASLLLAKGKKNLNG

NLFTGINNLDEGFIFTDATIQNDTISDQSQRVSQFDRLLAGLFPYITSEERQAVAKQYPI

SDAPSKGNTFSRISAVIADSTFVCPTYWTAEAFGSSAHKGLFDYAPAHHATDNSYYIGSI

WNGKKSVSSVQSFDGALGGFIETFNPNNNAANKTINPYWPTFDSGKQLLFNTTTRDTLSP
                                          |->Stop site
ADPRIVETSSLTDFGTSQKTKCDFWHGSISVNAGLQASF
```

FIG. 7

BNSDOCID: <WO__9812345A1_I_>

Base composition from 1 to 579
TRN 2-1738 RHODOSPORIDIUM ESTERASE cDNA

	Total	Percent
A	70	12.1
C	7	1.2
D	25	4.3
E	16	2.8
F	36	6.2
G	49	8.5
H	10	1.7
I	21	3.6
K	25	4.3
L	49	8.5
M	4	0.7
N	35	6.0
O	1	0.2
P	31	5.4
Q	26	4.5
R	15	2.6
S	53	9.2
T	43	7.4
V	32	5.5
W	10	1.7
Y	21	3.6
Acidic	41	7.1
Basic	40	6.9
Charged	81	14.0
Net charge	-1	-0.2
Hydrophobic	138	23.8
Residues	579	
MW	61875	

FIG. 8

BNSDOCID: <WO__ 9812345A1 I >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16193

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12P 21/06; C12N 1/00, 9/16, 15/00
US CL :435/69.1, 196, 325, 243, 320.1.
According to Internati nal Patent Classification (IPC) r to both national classificati n and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classificati n symbols)

U.S. : 435/69.1, 196, 325, 243, 320.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y ---- A	US 5,512,454 A (USHER et al.) 30 April 1996, see column 14, paragraph 5 and Examples 1 to 4.	27 ---- 14 ---- 1-13, 15-26, 28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 JANUARY 1998	Date of mailing of the international search report 27 JAN 1998
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